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Assaying Potential Carcinogens with Drosophila*

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Drosophila offers many advantages for the detection of mutagenic activity of carcinogenic agents. It provides the quickest assay system for detecting mutations in animals today. Its generation time is short, and Drosophila is cheap and easy to breed in large numbers. The simple genetic testing methods give unequivocal answers about the whole spectrum of relevant genetic damage. A comparison of the detection capacity of assays sampling different kinds of genetic damage revealed that various substances are highly effective in inducing mutations but do not produce chromosome breakage effects at all, or only at much higher concentrations than those required for mutation induction. Of the different assay systems available, the classical sex-linked recessive lethal test deserves priority, in view of its superior capacity to detect mutagens. Of practical importance is also its high sensitivity, because a large number of loci in one fifth of the genome is tested for newly induced forward mutations, including small deletions.

The recent findings that Drosophila is capable of carrying out the same metabolic activation reactions as the mammalian liver makes the organism eminently suitable for verifying results obtained in prescreening with fast microbial assay systems. An additional advantage in this respect is the capacity of Drosophila for detecting short-lived activation products, because intracellular metabolic activation appears to occur within the spermatids and spermatocytes.

Introduction

Recent results with microbial assay systems, such as the Ames test with Salmonella, have demonstrated a striking overlap between carcinogenicity and mutagenicity. That is, the great majority of compounds capable of producing malignant transformation are also effective in producing genetic changes in the form of heritable mutations. The possibilities the Ames test has to offer for a quick identification of potential carcinogens in the human environment is indeed a major accomplishment. Regulatory measures, with all the consequences for society, should, in our opinion, not be based on data obtained with a single assay system, but would require the confirmation after the application of a battery of different test systems. Since man is more closely related to other mammals than bacteria, various authors (*1,2*) emphasize the importance of mammalian assay systems. From a toxicological point of view, the intact mammal obviously offers enormous advantages over many other assay systems, since information regarding pharmacokinetics, as resorption, elimination, and drug metabolism both inside and outside the target cells, can only be obtained by studies on mammals. With regard to the genetic data that can be obtained from the intact mammal, we tend to be more pessimistic, however.

From the point of view of genetic hazards, transmissible point mutations and small deletions deserve probably the highest priority (*3*), particularly so since the induction of these changes correlates with the risk of carcinogenesis. For the detection of this class of genetic damage one has until now to rely entirely on specific locus tests. In view of the extreme costs and labor involved,

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there are only a few laboratories in the world where these can be carried out. The time and cost required to collect meaningful data places severe limitations on the number of chemicals and concentrations that can be investigated. Experimentation is restricted to high concentrations, and since dose-effect curves with chemical mutagens do not exhibit simple proportionality, there is no *a priori* justification for linear extrapolation to low concentrations.

All other *in vivo* mammalian assay systems which can be employed for routine screening purposes rely on the detection of chromosome breakage effects, whether these are recovered from bone marrow, peripheral blood, testes, or in dominant lethal assays. Recent experiments with Drosophila have revealed that a number of substances, the indirectly-acting carcinogens in particular, are highly effective in inducing mutations but do not produce chromosome breakage effects at all or induce breakage effects only at much higher concentrations than those required for mutation induction. Such compounds thus could spuriously register as safe in any routine mammalian *in vivo* assay system. Since it is particularly these substances which entail the risk of producing malignancy, mammalian tests relying on chromosome breakage cannot be considered as diagnostic. Salmonella, yeast, and mammalian cell cultures with microsomal extracts or Drosophila would seem preferable.

Advantages of Drosophila as a Test System

In the light of these considerations, the possibilities afforded by Drosophila for detecting various classes of mutagens will be discussed (*4-6*). In contrast to most other assay systems, where only one class of genetic damage can be studied, special tester strains permit the simultaneous assessment of the total spectrum of genetic changes, ranging from recessive lethal or visible mutations, small deletions, translocations, chromosome loss, and dominant lethals to nondisjunction and genetic recombination. This provides opportunity for a quantitative comparison of the detection capacity for various genetic end points. Before proceeding to a more detailed discussion of this topic, we want to elaborate on the capacity of metabolic activation, which makes Drosophila particularly suitable for the verification of the results with indirect carcinogens, as obtained in microbial assay systems.

Metabolic Activation by Insect Microsomes

Indirect mutagens and carcinogens require activation by the microsomal enzyme systems present in the mammalian liver. In microbial test systems, mutagens of this kind register as negative, unless host-mediated assays or plating on microsomal extracts from mammalian tissues are employed. Recent discoveries indicate that insect microsomes are capable of facilitating the same enzymic reactions as those from the mammalian liver. The endoplasmic reticulum is considered the center for drug and pesticide metabolism, and the enzymes involved are mixed-function oxidases. Unlike the mammalian liver, insects do not have any specific organ in which those enzymes are predominantly located. Biochemical characterization of the microsomal enzymes in insects has demonstrated that numerous foreign compounds are oxidatively metabolized by isolated microsomes or other subcellular fractions. The nature and diversity of the reactions catalyzed indicate that insect microsomes exhibit a similar degree of metabolic versatility and substrate nonspecificity as those from mammalian liver (*7*). Among the various types of reactions catalyzed are, for example, oxidative attack of aromatic rings, *C*-alkyl, *N*-alkyl, *S*-alkyl, or *O*-alkyl groups, all fundamental steps that are well known to play a part in the bioactivation of indirect carcinogens and mutagens. Most of the more direct biochemical information is derived from studies with the housefly, *Musca domestica*. The evidence that Drosophila is capable of facilitating similar reactions is based on mutation studies carried out mainly by Vogel during the last few years (*6*).

Some forty compounds that require metabolic activation have now been tested in Drosophila and yielded positive response for the induction of recessive lethals (see Table 1). First demonstration of biotransformation was obtained by Clark (*8*) for the pyrrolizidine alkaloid, heliotrine, and by Pasternak (*9-11*) for the dimethyl and diethylnitrosamines. More recently, Vogel has studied the response of Drosophila for various groups of indirectly-acting carcinogens. His data, and those of earlier studies, show that some 40 compounds falling into nine different groups, produce a positive response in tests for recessive lethal mutations with Drosophila (*6*). The genetic activity of these widely different substances convincingly demonstrate the versatility and lack of substrate specificity of the Drosophila enzyme

Table 1. *Mutagenic response of Drosophila to indirect carcinogens.*

Chemical class	No. tested	Mutagenic activity
Triazenes	12	+
Nitrosamines	6	+
Hydrazo-, azoxyalkanes	3	+
Oxazaphosphorines	3	+
DDT, DDA	2	+
Hempa	1	+
Aflatoxins	1	+
Pyrrolizidine Alkaloids	11	+
Vinyl chloride, chloroprene and related compounds	4	+
4-Dimethylamino-*trans*-stilbene	1	+
4-Nitroquinoline *N*-oxide	1	+
	45	

systems. Other convincing proof for the capacity of mammlian-like metabolic activation of *Drosophila* is provided by the finding of Pasternak (*10*) that out of six nitrosamines tested, only *N*-nitrosoethly-*tert*-butylamine was nonmutagenic. This substance was likewise noncarcinogenic in rats (*12*)and nonmutagenic in *E. coli* with or without rat liver microsomes (*13*).

Stage Specificity of Indirect Mutagens

One of the characteristic features of chemical mutagens is their specificity of action. Failure to detect mutagenic activity may result from pronounced stage-specific response. An additional advantage *Drosophila* has to offer is the facility with which mutagenic activity can be sampled in a wide variety of different germ cell stages. In fact, one of the classical examples of specificity at the cellular level is provided by Auerbach's observations (*14*) for formaldehyde. Sensitivity differences between germ cells can be ascertained by mating the treated males to fresh females in a succession of different broods, or in females by setting up successive subcultures.

In the studies on the mode of action of indirect mutagens by Vogel, the application of this brooding technique has revealed the interesting observations that indirectly acting carcinogens exhibit a similar stage-specific sensitivity pattern, despite considerable differences in their mode of activation (see Fig. 1). That is, spermatids and spermatocytes appear to show greatest response (*6,15,16*). Thus representatives of four different groups of indirect mutagens, that is, PDMT (1-phenyl-3, 3-dimethyltriazene) 2,4,6-TCl-PDMT,

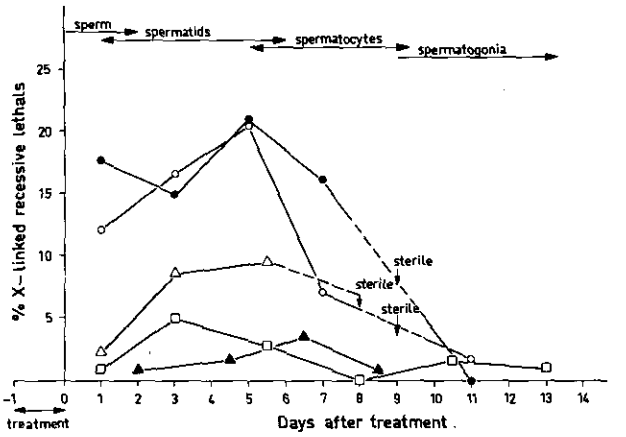


FIGURE 1. Stage-specific induction of recessive lethals by various indirectly acting mutagens in Drosophila males after feeding for 24 hr: (O) 2,4,6-TCl-PDMT; (●) PDMT (phenyl dimethyltriazene); (△) azoxymethane; (□) DEN; (▲) cyclophosphamide.

diethyl nitrosamine, azoxymethane, and cyclophosphamide all exhibit peak activity 4-6 days after treatment and reduced fertility after 7-9 days. From studies with the electron microscope by Tates (*17*), it is known that in these stages the endoplasmic reticulum is most highly developed. Since this system is regarded as the site of metabolic activation, these observations provide an indication for intracellular activation within these particular stages of sperm development. Thus, even in situations where short-lived activation products are involved, with mutagenic effects restricted to the cells in which activation had occurred, Drosophila will still permit their detection. Such effects may well be missed in host-mediated or *in vitro* microsome-facilitated assay systems; the experiences with the nitrosamines in other test systems seem to present an example in case.

Comparison of Endpoints and Validation of Assay Systems

A crucial point in the analysis of potential mutagens by use of Drosophila is the selection of the most suitable assay system for the detection of genetic effects. Drosophila permits the simultaneous assessment of the total spectrum of genetic effects. As part of a screening program in a whole battery of different test systems, it will not be possible to assay in Drosophila for all genetic changes that might be analyzed. Obvious-

ly, a proper choice has to be made between the various techniques and tester strains available.

The relevant criteria applied require that the tests employed should possibly be nonspecific and capable of detecting a wide range of mutagens, and that they permit the detection of genetic damage relevant to man. In brief, what we need to know is what test system will be most reliable in detecting potential mutagens. Pertinent questions thus are the extent to which induction of one type of genetic change correlates with the occurrence of another, and the incidence for a given mutagen, at different concentrations, of different kinds of genetic changes. To this end Vogel et al. (*16,18*) carried out a comparative study after feeding different mutagens to males, determining the frequency of induction at various concentration levels of recessive lethals, dominant lethals, and chromosome loss (and in some instances translocations).

For the presentation of the results of these experiments, in summarized form, a comparison for the different genetical end points is given in terms of the ratio between the lowest effective concentration (LEC) and LD_{50}. LEC is defined as the lowest concentration of a chemical which causes a significant increase of the mutation or breakage frequency above that in the controls (*18*). This estimate provides evidence of the ability of a mutagen to induce various kinds of genetical damage at toxic, subtoxic, and very low dose levels. It is this kind of information which is obviously needed for the evaluation of assay systems.

From a comparison of the results obtained with MMS (methyl methanesulfonate), TEB (tetraethyleneiminobenzoquinone), 2,4,6-TCl-PDMT and DEN (diethylnitrosamine) it became clear that dominant lethals and chromosome loss cannot be detected at low concentrations, except when the polyfunctional agent TEB is used (see Table 2), MMS, for example, gives a LEC:LD_{50} ratio for recessive lethals of 1:100, but 10-fold or even 20-fold higher concentrations are required for inducing significant numbers of dominant lethals and chromosome loss, respectively. For the triazene, 1/1000 of LD_{50} is sufficient for recessive lethal induction, but 1:4 and 1:2 are the ratios calculated for dominant lethals, chromosome loss and translocations. These data clearly demonstrate that the resolving power for detecting potent mutagens with Drosophila depends on the genetic endpoint applied, and this finding is of significance when evaluating testing procedures. The observation that lowest LEC values and thus highest mutagenic effectiveness was recorded for recessive sex-linked lethals, clearly indicates the superior detection capacity of this simple classical assay system for the detection of mutagens. It is of practical importance, that by using the whole X-chromosome about one fifth, that is a sizable section of the genome, is included in the test. The large number of loci which are tested in this way guarantee for the high sensitivity of the recessive lethal test. Compared to the mouse, this is indeed a tremendous advantage (*19*). Since, as has been stated above, recessive lethals do include small deletions, these findings are of significance in the detection of this most important class of genetic damage.

Another interesting finding in these studies is that diethyl nitrosamine (*5mM*) is highly effective in inducing recessive lethals of up to 25% in the X-chromosome, in fact mutating more than 100% of the genome. When considering that detrimentals may be 10-20 times as frequent as recessive lethals (*20*), the mutagenic potential of this compound is indeed formidably high. By contrast, at the same concentration, no chromosome breakage effects are produced at all. The results, in this respect, are similar to those obtained with diethyl sulfate (*21-26*) and hycanthone (*27*). Mutagens of this kind would be difficult to detect in any assay system in which chromosome breakage is applied as the only criterion of mutagenic activity. For dominant lethal induction in rats and mice, DEN and dimethyl sulfate (DMS), related to diethyl sulfate, were indeed ineffective (*28, 29*): similarly negative results were recorded for dimethylnitrosamine (DMN) and *N*-nitrosomorpholine (*30*), though they are highly effective in producing mutations in Drosophila.

The observation that mutagens like MMS and TEB produce only chromosome aberrations at considerably higher concentrations than those required for recessive lethal induction is likewise of interest in assessing the detection capacity of various assay systems. Findings like these raise the possibility that, when assay systems relying on chromosome breakage only, as is, in fact, the case for all routine mammalian *in vivo* tests, whether these be dominant lethals, chromosome aberrations in bone marrow or peripheral blood, translocations or micronuclei, false negatives can be obtained, simply because the concentration at the target cells is not sufficiently high.

An indication of such phenomena has been obtained by Vogel when comparing the mutagenic

Table 2. LEC:LD_{50} ratio in Drosophila sperm for various genetic endpoints.

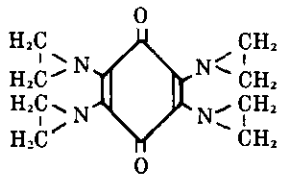
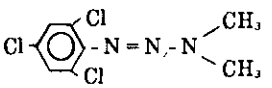
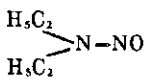
Compound	Recessive lethals	Dominant lethals	Chromosome loss		Translocations (II-III)
			Entire(X,Y)	Partial (Y^L,Y^S)	
$CH_3SO_2OCH_3$ (MMS)	1:00	1:10	1:10	1:5	No test
 (TEB)	1:5000	1:1000	1:200	1:200	1:1000
 (2,4,6-TCl-PDMT)	1:1000	1:4	1:2	–	1:4
 (DEN)	1:100	–	–	–	–

Table 3. Specific activity of indirect mutagens (carcinogens) in Drosophila.

Chemical	Recessive lethals	Dominant lethals	Chromosome loss		Translocations (II-III)
			Entire (X,Y)	Partial (Y)	
DEN	+	0	0	0	0
2,4,6-TCl-PDMT	+	(+)?	(+)	0	(+)
3-PyDMT	+	0	No test	No test	No test
Cyclophosphamide	+	0	0	0	No test
Trofosfamide	+	0	0	0	No test
Ifosfamide	+	0	0	0	No test

effectiveness of 1-(pyridyl-3)-3,3-dimethyltriazene (3-PyDMT), and three oxazaphosphorines, cyclophosphamide, ifosfamide, and trofosfamide (Table 3). For these substances, recessive lethal induction is concentration-independent; that is, the recessive lethal yield never exceeds 1-3% (*31*). This observation is considered as an indication that the amount of active metabolites at the target is restricted, by a rate-limiting factor in the metabolism of these indirect mutagens. Chromosome breakage effects are not observed after the application of these four mutagens. However, one should bear in mind that with powerful mutagens like MMS, TEB, and PDMT, no chromosome loss and dominant lethals are observed at concentrations yielding recessive lethal frequencies equivalent to those obtained for PyDMT and the three oxazaphosphorines. Substantially higher concentrations were in fact required to obtain a significant increase. When considered together, these findings suggest then that because of the existence of a rate-limiting factor, or of the extreme toxicity of the compounds in question, a potent chromosome breaker may remain undetected.

There is another reason, we believe, why in prescreening for carcinogens the tests for mutagenicity deserve priority to those relying on chromosome breakage. And this is that as a primary effect there does not seem to be a causal relationship between the carcinogenic potential and the induction of chromosome aberrations.

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